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Report

Active Process Mediates Species-Specific Tuning of *Drosophila* Ears

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Summary

The courtship behavior of Drosophilid flies has served as a long-standing model for studying the bases of animal communication [1]. During courtship, male flies flap their wings to send a complex pattern of airborne vibrations to the antennal ears of the females. These "courtship songs" differ in their spectrotemporal composition across species and are considered a crucial component of the flies' premating barrier [2, 3]. However, whether the species-specific differences in song structure are also reflected in the receivers of this communication system, i.e., the flies' antennal ears, has remained unexplored. Here we show for seven members of the *melanogaster* species group that (1) their ears are mechanically tuned to different best frequencies, (2) the ears' best frequencies correlate with high-frequency pulses of the conspecific courtship songs, and (3) the species-specific tuning relies on amplificatory mechanical feedback from the flies' auditory neurons. As a result of its level-dependent nature [4, 5], the active mechanical feedback amplification is particularly useful for the detection of small stimuli, such as conspecific song pulses, and becomes negligible for sensing larger stimuli, such as the flies' own wingbeat during flight.

Results and Discussion

In order to find a mate, most sexually reproducing animals must be able to distinguish males from females and conspecifics from heterospecifics. To this end, complex multisensory communication systems have evolved [6, 7] that serve the fundamental tasks of sex and species recognition [8, 9], as well as the more advanced task of assessing the fitness and reproductive value of a potential mate [10, 11]. Auditory information, in the form of species-specific sound signals, plays an important role in the reproductive behavior of both vertebrates and insects [12–18]. A prominent example includes the acoustic courtship of flies of the genus *Drosophila* [3, 19–21]. As part of the mating ritual, the males vibrate their wings to send a species-specific sound signal to the females. These courtship songs, which have been reported for over 100 *Drosophila* species [22], are widely considered to be a crucial component of the flies' premating barriers, contributing to both reproductive isolation and speciation in Drosophilid flies [23, 24]. However, whether the flies' antennal ears are spectrally tuned to the composition of conspecific songs has not been explored yet. Here we present a comparative study of antennal tuning and sound emissions in seven members of the *melanogaster* species group [25].

Active Feedback Mediates Species-Specific Antennal Tuning in *Drosophila*

In the first step, we analyzed the mechanical properties of the flies' ears. The *Drosophila* antennal ear is composed of two functional units (Figure 1A, inset): the pedicellus, which harbors the mechanosensory neurons of Johnston's Organ (JO) [26], and the funiculus, which, together with its lateral arista, acts as the fly's sound receiver. In response to near-field sound, the funiculus starts rotating back and forth about its longitudinal axis [27], thereby directly gating mechano-transducer channels (METs) in the membranes of JO neurons to which the funiculus is mechanically coupled [28]. As a result of this construction, the mechanical interactions between sound receiver and METs become inherently reciprocal: just as the movements of the receiver modulate the open probabilities of METs, the gating of METs affects the receiver's mechanics. Experimentally, these relations can be exploited to deduce auditory response properties from analysis of sound receiver motion [4, 5, 29]. Here we used a laser doppler vibrometer to record movements of the flies' antennal ears in the absence of experimental stimulation. These free fluctuations, which betray the receivers' best frequencies for small disturbances, represent two phenomena: the receiver's passive motion due to the thermal bombardment by surrounding air particles and its active motion due to mechanical feedback from the auditory neurons [4, 29]. Similar to *D. melanogaster* [29], sound receiver motions of all experimental species were well described by a simple harmonic oscillator model (Figure 1A), indicating uniformity of the receivers' underlying gross anatomical design. The receivers differed, however, substantially in their best frequencies (Figure 1B, top), which ranged from 147 ± 20 Hz in *D. melanogaster* to 293 ± 52 Hz in *D. mauritiana* (see Table S1 available online; all standard deviations specify interindividual variation). To explore the origin of this species-specific auditory tuning, we anaesthetized the flies with CO_2, which, in *D. melanogaster*, has been shown to reversibly eliminate the active feedback from auditory neurons [5]. CO_2 anesthesia thus provides an experimental tool to distinguish between active and passive tuning mechanisms. Consistent with a loss of transducer-based feedback amplification, the amplitudes of the receivers' free fluctuations decreased and the receivers' best frequencies increased in the ears of CO_2-sedated flies. Also, the relative range of receiver tuning across species was reduced, with best frequencies ranging from 789 ± 45 Hz in *D. melanogaster* to 991 ± 47 Hz in *D. ananassae* (Figure 1B, bottom; Table S1). These findings suggest that hearing in all experimental flies is supported by the same active, transducer-based process previously described for

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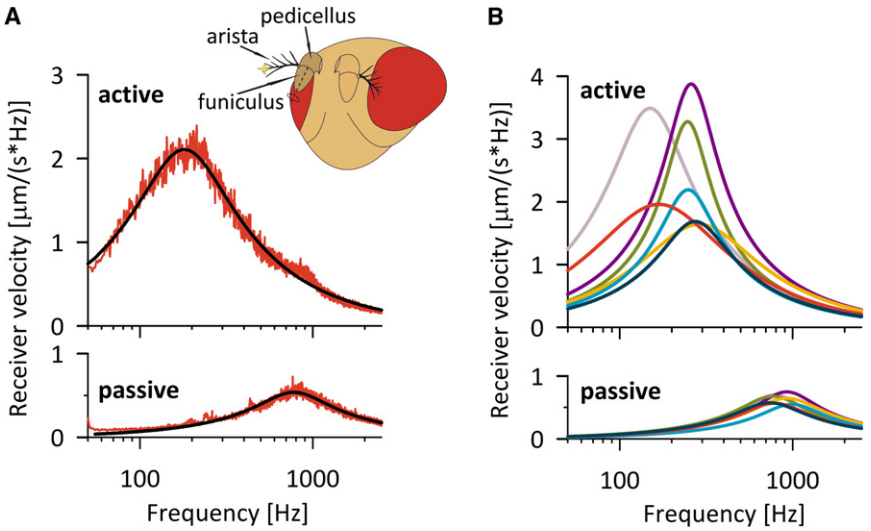


Figure 1. Species-Specific Frequency Tuning of *Drosophila* Antennal Ears

(A) The free mechanical fluctuations of the antennae are well described by a simple harmonic oscillator model both in the active receivers of awake (top) and in the passive receivers of CO_2-anaesthetized (bottom) flies. Example shows data (red) and model (black) for an individual receiver of *D. teissieri*. Inset: functional anatomical sketch of the *Drosophila* antennal ear (asterisk marks point of vibrometric measurements).

(B) Mean fits of harmonic oscillator model to the receiver fluctuations of awake (top) and CO_2-anaesthetized (bottom) flies for all experimental fly species: *D. simulans* (purple), *D. mauritiana* (yellow), *D. melanogaster* (gray), *D. teissieri* (red), *D. yakuba* (green), *D. erecta* (dark blue), and *D. ananassae* (light blue). The same color code has been used throughout the figures.

D. melanogaster [4, 5, 29] and that this active process is essential for the species-specific tuning of the flies' ears.

High-Frequency Content of *Drosophila* Courtship Songs Differs across Species

In the second step, we recorded the flies' courtship songs (Figure 2). In accord with previous studies, all detected song elements could be classified as either sine songs or pulses. Sine songs represent continuous, pure-tone-like waveforms with a typical duration in the order of seconds; they were absent in *D. yakuba* and *D. ananassae*. Pulses represent shorter waveforms with a typical duration in the order of milliseconds, which are usually repeated to form trains, the so-called "pulse songs." Pulse songs can be characterized by two major descriptors: the principal frequency component of the individual pulse (intrapulse frequency, or IPF) and the time interval between pulses within a train (interpulse interval, or IPI). It is generally agreed that IPIs, which in our recordings ranged from 370 ± 95 ms in *D. erecta* to 14 ± 1 ms in *D. ananassae* (see also Table S2), differ across species and that they constitute a key factor in species recognition [29–31]. However, if in the acoustic communication system of *Drosophila* it is the brain's prime task to detect conspecific pulse trains by analyzing the intervals between individual pulses, then it should be the ear's prime task to detect the individual pulses in the best possible way. We therefore analyzed the spectral composition of the flies' pulses in closer detail and specifically probed for possible correlations with the tuning of the conspecific receivers. Because both the spectral composition of *Drosophila* courtship song pulses [32–34] and the frequency content of active oscillations in the antennal ears of mosquitoes [35] have previously been shown to vary with temperature, we took great care that all experiments were carried out under the same conditions (23°C ± 1°C and 50% relative humidity). Consistent with previous reports [36], some species produced two or three different types of pulses, which were clearly distinguishable by both their waveforms and spectral profiles (see Figure 2 and Table S1). The spectral analysis of the short, and broadband, pulses is less straightforward than that of the long, and narrowband, sine songs. In order to extract the pulses' IPFs, we calculated the median frequency of the fast Fourier transform (FFT) spectrum of a rearranged pulse train (see Supplemental Experimental Procedures for details), which often coincided with the actual maximum of the FFT. Across, and in some cases also within, species, the different pulse types covered a wide range of IPFs, ranging from 138 ± 9 Hz for the lowest frequency pulse in *D. yakuba* to 423 ± 53 Hz for the highest frequency pulse in *D. mauritiana* (Table S1). Sine songs, in contrast, spanned a much narrower band of frequencies ranging from 136 ± 18 Hz in *D. melanogaster* to 187 ± 34 Hz in *D. erecta* (Table S1).

Receiver Tuning Is Correlated with High-Frequency Pulses of Conspecific Courtship Songs

From an auditory point of view, the occurrence of high-frequency pulses is especially interesting. In *D. melanogaster*, active mechanical feedback from auditory neurons shifts the sound receiver's best frequency from ~800 Hz, representing the passive receiver, to below 200 Hz [4, 5], concomitantly boosting its mechanical sensitivity in the frequency range of male sound emissions [5]. As a result of this frequency-specific amplification, the sensitivity of the active receiver decreases steeply for frequencies above ~200 Hz [5]. The use of sound signals with frequencies considerably higher than 200 Hz by other *Drosophila* species may thus necessitate an upward shift in the best frequencies of their active ears. We have tested whether the observed differential tuning of the flies' antennal ears (Figure 1B) reflected these requirements. The frequency tuning of the flies' ears was indeed strongly correlated with the highest frequency pulses occurring in the conspecific songs (Spearman's $\rho = 0.96$, $p = 0.003$; Figure 3A, left). The tuning correlation was lost when the same correlation analysis was carried out for the passive ears of CO_2-sedated flies (Spearman's $\rho = 0.14$, $p = 0.78$; Figure 3A, right), demonstrating its dependence on active mechanical feedback from the flies' auditory neurons. These findings not only identify a material substrate for species-specific acoustic communication, namely the core modules of auditory mechanotransduction (i.e., the functional complex of transducer channels, gating springs, and molecular motors), but also lead us to suggest that species-specific ranges of audibility may help to maintain reproductive isolation in Drosophilid flies. In evolutionary

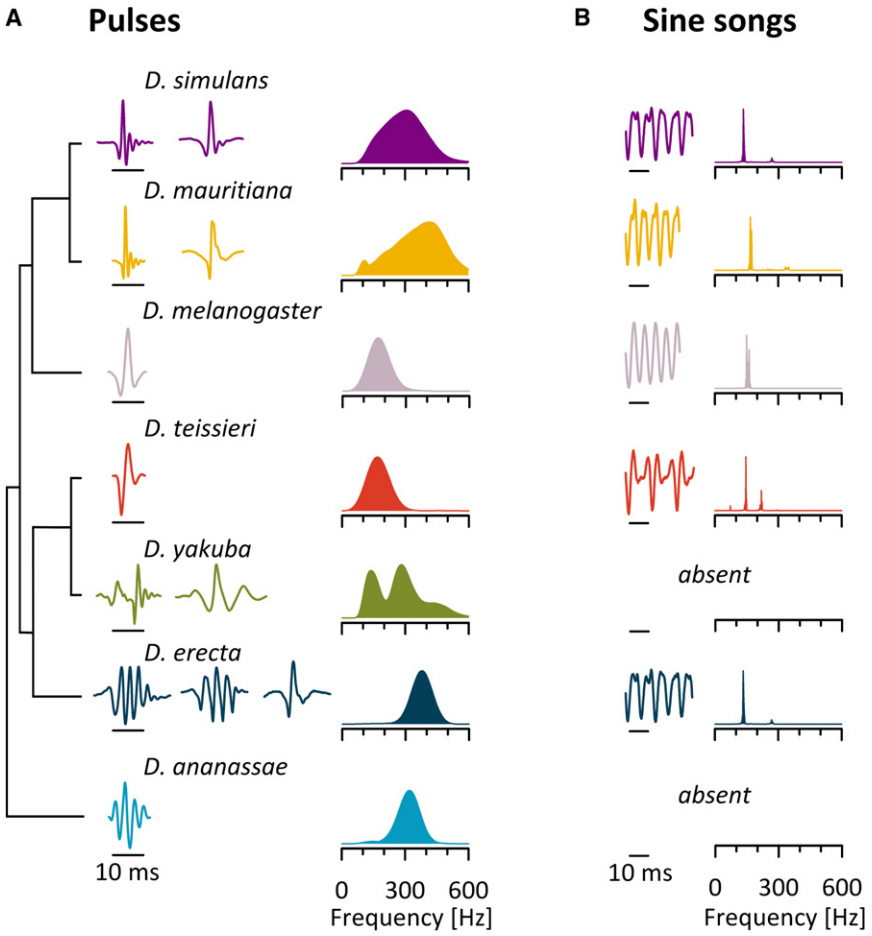


Figure 2. Components and Spectral Composition of Courtship Songs

(A) Cladogram depicting phylogenetic relationships between experimental flies (left; combined from [52, 60]), exemplary time traces for the pulse types occurring in the respective courtship songs (middle), and details of the spectral composition of the pulse type with the highest intrapulse frequency (right). Color code (see Figure 1B) applies to all panels.

(B) Exemplary time traces (left) and spectral composition (right) of the sine songs.

terms, the experimental flies of this study are still a very young group, and crossbreeding between some of the species still leads to viable, though sterile, hybrids [30]. Moreover, several members of the *melanogaster* species group, such as the two species pairs *D. melanogaster/D. simulans* and *D. teissieri/D. yakuba*, have been found to occur sympatrically, sharing the same feeding and breeding sites [31]. Taken together, these conditions may build up a considerable selective pressure for the evolution of premating isolation mechanisms. Sympatric diversification of both signal composition and signal preference has previously been reported in the acoustic communication systems of tree [32] and chorus [33] frogs. Our results now suggest that, in *Drosophila*, one mechanism of adjusting the receivers' signal preference may involve the species-specific tuning of their ears to conspecific high-frequency sounds (Figure 3).

Active, Nonlinear Feedback Amplification Boosts Antennal Response to Song Pulses but Becomes Negligible during Flight

In the insect order Diptera, which comprises "common flies" along with mosquitoes [34], acoustic communication and flight behavior are intimately linked [20]. In *Drosophila*, the groups of wing muscles that generate and control the aerodynamic forces during flight have also been implicated in the spectrotemporal patterning of the courtship song [35, 36]. In mosquitoes, in which the males find their females phonotactically by using the females' flight tones as an acoustic cue [37], singing and flying are essentially the same act [38], and the males' antennae are therefore closely tuned to the females' wingbeat frequencies (WBFs) [39, 40]. Interestingly, wing movements during *Drosophila* courtship have been interpreted as a ritualized form of flight, and an overlap of the central pattern generators underlying the two types of behaviors has been claimed [36]. To entangle matters further, the fly's antenna, next to playing a role in courtship behavior [41–43], has also been implicated as a sensor within the insect's flight control loop [44, 45]. In order to test whether the flies' antennae are directly tuned to the WBFs, we recorded sound emissions (Figure 4A and Table S3) during tethered flight for all seven experimental species. The WBFs, as measured by the principal frequency components of flight-induced sound emissions, ranged between 145 ± 20 Hz in *D. melanogaster* and 213 ± 15 Hz in *D. mauritiana* (Table S3). This is close to the spectral range covered by the sine songs. WBFs were in positive correlation with the best frequencies of the fluctuations of the active antennae (Spearman's $\rho = 0.93$, $p = 0.0067$; Figure 4B, left), but not with those of the passive antennae (Spearman's $\rho = 0.11$, $p = 0.83$; Figure 4B, right). In *Drosophila*, mechanical feedback amplification and resulting antennal tuning are level dependent: whereas the free fluctuations of the active antenna predict the best frequency for small stimuli, the free fluctuations of the passive antenna predict its best frequency for large stimuli (see Figure 4C and [46]). To assess the physiological relevance of the observed correlation between WBFs and active antennal tuning, we measured the magnitudes of wingbeat-evoked antennal deflections. The peak-to-peak amplitudes of antennal displacements ranged between 10.4 ± 3.9 μm in *D. ananassae* and 20.3 ± 10 μm in *D. teissieri* and were thus several orders of magnitude higher than the active antennae's free fluctuation twitches ([29] and data not shown). At such high displacement amplitudes, the contributions of mechanical feedback amplification to the antennae's response properties are expected to become negligible, and the antennal oscillators are likely to be determined exclusively by their passive properties. We tested, and confirmed, this expectation by exposing the antennae of *D. teissieri* to various stimuli of a range of different intensities. Using pulse-like,

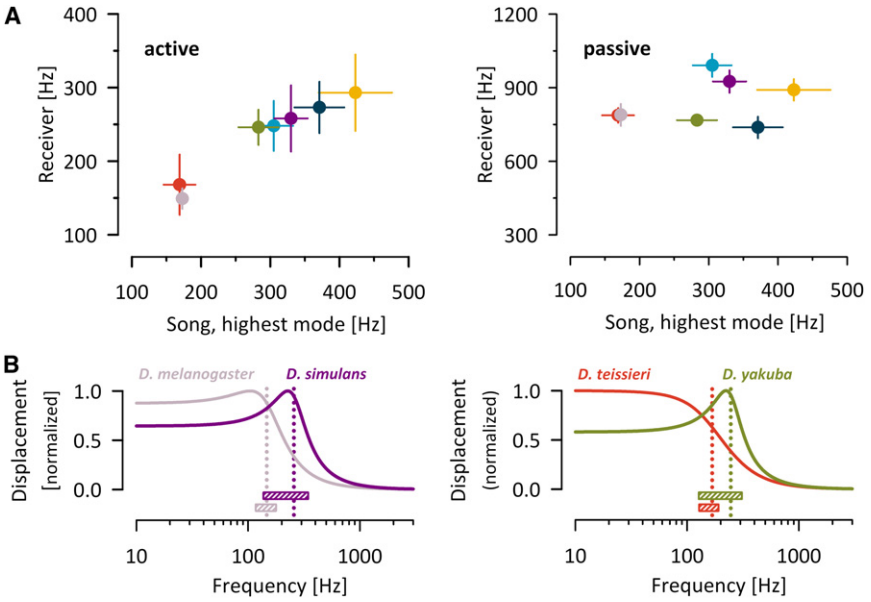


Figure 3. Active Process Tunes Ears to Conspecific High-Frequency Sound Emissions

(A) Receiver tuning correlates with the highest-frequency pulses of the conspecific courtship song in the active receivers of awake flies (left, Spearman's $\rho = 0.96$, $p = 0.003$). Tuning match is abolished in the passive receivers of CO_2-anaesthetized flies (right, Spearman's $\rho = 0.14$, $p = 0.78$). Error bars indicate interindividual variation (±1 standard deviation [SD]).

(B) Displacement responses for weak stimuli (shown as the mean fit of a harmonic oscillator model to the receivers' free mechanical fluctuations) for the antennal ears of two sympatrically occurring species pairs: *D. melanogaster*/*D. simulans* (left) and *D. teissieri*/*D. yakuba* (right). Vertical, dotted lines mark the receivers' best frequencies; horizontal, dashed bars mark the spectral range of conspecific sound emissions.

wingbeat-like, and broadband stimulation (Figure 4C), we found high displacement gains of ~8 ($displacement_{active}$/$displacement_{passive}$) corresponding to best frequencies of around 150 Hz for small deflections (<300 nm, peak to peak) but displacement gains of only ~1 corresponding to best frequencies of around 800 Hz for large deflections (>10 μm). The stimuli that are produced during tethered flight, leading to antennal deflections of ~20 μm (peak to peak; Table S3), are thus sufficiently strong to drive the antenna into the passive regime. The stimulus load during real flight, however, is likely to be even higher. During real flight, the forces resulting from the flies' wingbeat will be joined by the forces caused by the attacking flight wind and inertial forces arising from angular or linear accelerations. Taken together, these data strongly suggest that in the flying animal, the active transducer-based process that provides mechanical feedback amplification to the antennal movements becomes negligible, leaving the antennae dominated by their passive properties. The correlation between the best frequencies of the active antennae's fluctuations and the WBFs therefore does not represent an adaptive cotuning, as described for mosquitoes [39, 40], but is likely to reflect a more indirect effect. One possible source of the observed correlation might be the shared

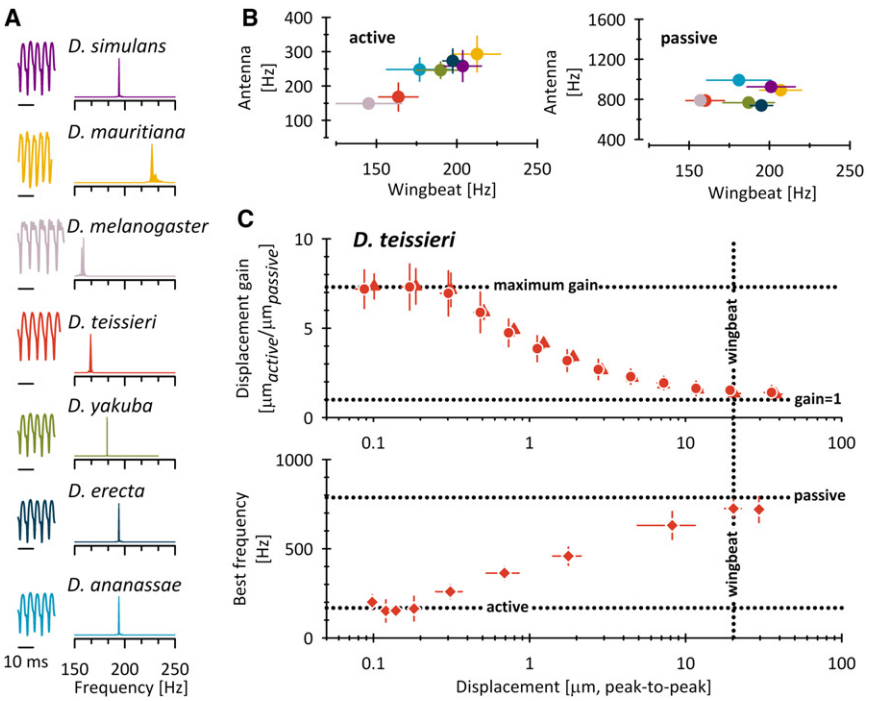


Figure 4. Active Antennal Tuning Facilitates the Detection of Weak Conspecific Sound and Becomes Negligible during Flight

(A) Exemplary time traces (left) and spectral composition (right) of the flies' sound emissions during tethered flight.

(B) Antennal tuning correlates with the wingbeat frequencies during flight in the active receivers of awake flies (Spearman's $\rho = 0.93$, $p = 0.0067$) but not in the passive receivers of CO_2-anaesthetized flies (Spearman's $\rho = 0.11$, $p = 0.83$).

(C) The antennae of *D. teissieri* were stimulated with pulse-like (1.5 periods of a 169 Hz waveform, triangles), wingbeat-like (ten periods of a 164 Hz waveform, circles), and broadband (multisine from 12 Hz to 3192 Hz, diamonds) stimuli. The large, flight-evoked stimuli (vertical line; see also Table S2) drive the antennae into the passive regime, which is characterized by decreased displacement gains, with values close to 1 (lower horizontal line, top), and increased best frequencies, with values of ~750 Hz, close to the best frequencies of the passive antennae (upper horizontal line, bottom). Stimulation with smaller stimulus magnitudes, in contrast, yields maximal displacement gains of ~7.5 (upper horizontal line, top) and best frequencies of ~150 Hz, close to the best frequencies of the freely fluctuating, active antennae (lower horizontal line, bottom). All error bars indicate interindividual variation (±1 SD).

neuromuscular substrate for song production and flight. In this scenario, higher-frequency sound emissions would be mechanistically linked to, and thus directly correlated with, higher WBFs.

Conclusions and Outlook

It is a fundamental requirement of all communication systems that the physical properties of the receivers reflect, and ideally match, the physical properties of the signals emitted by the senders [47]. Studies on passerine birds [48], anuran amphibians [49], and orthopteran insects [15] have brought forth a vast body of knowledge about the general neuroecological conditions and signaling strategies used in animal acoustic communication. Both signaler/receiver matches—and occasional mismatches—have been detected and discussed in their respective ecological contexts [50, 51]. Little is known, however, about the genetic basis and molecular evolution of acoustic communication systems. Here we show for flies of the genus *Drosophila* that the frequency tuning of their antennal sound receivers correlates with high-frequency pulses in the conspecific courtship songs and that this species-specific tuning is likely to originate from the molecular modules for mechanotransduction. The particular link between sound receiver tuning and high-frequency signal components is feasible from a theoretical point of view: the displacement response of a moderately damped harmonic oscillator, such as the *Drosophila* antenna, decays steeply when forced above but remains rather flat when forced below its best frequency (see also Figure 3B). High-frequency signals (i.e., signals above the receivers' best frequencies) thus pose the greater challenge to the system and should lead the tuning. The fact that, for most of the species, the best frequencies of the receivers' free fluctuations remained lower than the actual highest-frequency pulses of their courtship songs may reflect a tuning trade-off between lower- and higher-frequency song elements. Because of the level dependence of mechanical feedback amplification (Figure 4C and [4, 46]), however, the best frequencies of the stimulated receivers will be higher, which, at least for moderate stimulus amplitudes, will improve the tuning match. Future studies are required to address the behavioral relevance of individual song components and especially the role of the faint, high-frequency pulses, which the flies' ears seem to be primarily tuned to. Also, it will be interesting to see how the differential tuning of the flies' ears has been implemented molecularly on the level of their mechanotransduction machineries and, finally, how this tuning contributes to the enhanced audibility of soft conspecific as compared to heterospecific sounds.

The reported tuning match between ears and songs in flies of the genus *Drosophila* (which contains 12 species with completely sequenced genomes [52]) offers a promising model platform to explore the evolutionary, mechanistic, and molecular bases of signaler/receiver coevolution. In the case of animal premating communications, the signaler/receiver cotuning is commonly deemed to arise from stabilizing sexual selection [53]: the strong selective pressure acting on an animal's mating decision will favor mutations that increase and penalize mutations that decrease the signal/receiver match. On the most elementary level, signals and receivers are thus indirectly coupled by being exposed to a common selective pressure. It was hypothesized almost half a century ago, however, that next to this indirect coevolutionary coupling, a second, more direct relation could exist that is commonly referred to as "genetic coupling" and is considered to arise from an overlap of the network of genes underlying sender and receiver function [54, 55]. The existing evidence, however, is inconclusive, and on the single gene level, no study has unambiguously demonstrated the existence of such a genetic coupling for any signaling system [53, 56, 57]. It appears promising to use the vast toolbox of *Drosophila* neuroethology to help shed light on this and other unanswered questions of acoustic communication.

For large stimuli, such as those produced during tethered flight, the species-specific antennal tuning is abolished, suggesting that the behaviorally (and evolutionarily) relevant signals of acoustic communication are comparatively small. The actual magnitudes of naturally occurring antennal displacements during courtship, however, are yet to be measured. In flying *Drosophilae*, antennal responses are determined by the linear properties of their passive mechanics, and active feedback amplification is thus irrelevant for the antennae's possible role in wingbeat control. This suggests an interesting dual mode of operation: during flight, the antennae operate in their passive, linear mode and well below their best frequencies (passive best frequencies range from 789 Hz to 991 Hz, and WBFs range from 145 Hz to 213 Hz for the species of this study). Here the resulting constant phase relation between antennal oscillations and wingbeats (see also Figure S1) is independent of variations of wingbeat amplitude, which should benefit the neural implementation of sensory-motor feedback loops. During acoustic communication, in turn, the antennae operate in their active, nonlinear mode and around the best frequencies of their receivers (active best frequencies range from 147 Hz to 293 Hz, and song emissions range from 127 Hz to 423 Hz for the species of this study), thereby specifically boosting their mechanical responses to soft sound in the frequency range of conspecific courtship songs (Figure 4C). Underlining the crucial role of sound intensity, previous playback experiments have indeed detected song level-dependent changes of *Drosophila* mating behavior, with courtship interactions being more frequent at low sound levels [58, 59]. In the end, when singing a love song to a fruit fly, it may be better to whisper than to shout.

Supplemental Information

Supplemental Information includes two figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.03.001.

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